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TITLE: Myeloid-Biased Stem Cells as Potential Targets for Chronic Myelogeneous Leukemia

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14. ABSTRACT

Purpose: CML results from the malignant transformation of a pluripotent hematopoietic stem cell (HSC). We identified novel subsets of HSC, called Myeloid-biased (My-bi) HSC. These HSC are epigenetically programmed to generate progeny that is skewed towards the myeloid lineage. Thus, both the normal My-bi HSC and the transformed CML HSC generate a myeloidbiased progeny. Accordingly, we hypothesized that My-bi HSC are the target of transformation that can lead to CML.
Scope: We are taking advantage of a mouse model for CML. We will isolate the different types of HSC that we have identified and will infect these with replication deficient retroviri containing the myeloid-associated p210 form of the Bcr/Abl construct. If our hypothesis is correct, My-bi HSC, but not balanced or Ly-bi, HSC can be transformed to give rise to myelogenous leukemia.
Up-to date Progress: We have begun to generate clonally repopulated host animals and have identified linage biased HSC. We have also begun a series of experiments to define the conditions that will yield high titer retrovirus for the proposed studies.
Significance: The proposed studies for the first time raise the possibility to selectively target My-bi HSC for therapy. This would leave other HSC untouched, limiting the toxicity of therapy.

15. SUBJECT TERMS
Hematopoietic stem cells, lineage potential, target of transformation, CML, BCR/ABL

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Exploration – Hypothesis Development Award (EHD) W81XWH-04-1-0798
Muller-Sieburg, Christa E

1 September 2004 -30 September 2005

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1. STATEMENT OF WORK as funded

Task 1: Are Myeloid-biased stem cells the target of transformation for CML? *Months 1-18*

A: Establish hosts repopulated with lineage-biased stem cells (HSC) using in vivo limiting dilution repopulating experiments with Sca-1+Lin- HSC.
B: Amplify plasmids containing the BCR/ABL p210 and p185 forms and produce high titer retrovirus
C: Isolate HSC from hosts that are repopulated by individual myeloid-biased (My-bi), lymphoid-biased (Ly-bi), or balanced repopulated hosts using existing animals (repopulated by clonally derived HSC isolated after in vitro limiting dilution)
D: Infect HSC with retroviri containing the p210 and p185 Bcr/Abl constructs and empty vector as a control.
E: Inject each type of infected HSC into 3 groups of new hosts: i) mock-infected, ii) p210 infected, iii) p185 infected.
F: Monitor appearance of donor type Leukemia in blood, BM and spleen, determine the lineage dominance of the Leukemia
G: Milestone: Verify or reject hypothesis: CML-like (myeloid-dominant) leukemia is generated when My-bi HSC, but not Ly-bi or balanced HSC, are transformed.

H: If the hypothesis is correct then repeat steps D-F with HSC identified from the animals created in step A to independently verify the hypothesis.

2. PROGRESS REPORT

Introduction

This project is designed to test the hypothesis that CML results from the transformation of a myeloid-biased hematopoietic stem cell (HSC). The normal HSC compartment consists of (at least) 3 subclasses of HSC, namely balanced, lymphoid-biased and myeloid biased HSC.[1,2] These types of HSC are defined by their ability to generate mature cells that can be detected in blood. Balanced stem cells generate about 10-20% mature myeloid cells and 80-90% mature lymphoid cells in blood. Lymphoid-biased HSC generate few myeloid cells but normal levels of lymphoid cells. Therefore, the peripheral blood leukocytes appear skewed towards the lymphoid lineage. Myeloid-biased HSC generate too few lymphoid cells and therefore the ratio of lymphoid to myeloid cells in blood appears to be skewed towards the myeloid lineage. All of these are true HSC, because they give rise to all lineages (albeit with skewed ratios) and they have self-renewal capacity. Indeed, the altered differentiation capacity is fixed on the level of the HSC through epigenetic imprinting.

In addition to the differences in lineage contribution, the different types of lineage biased HSC also differ in self-renewal capacity.[2] Myeloid-biased (My-bi) have the longest lifespan and a single clone of My-by HSC can sustain peripheral hematopoiesis for over 40 months. This is about 2 times the life span of a mouse. Lymphoid-biased (Ly-bi) HSC have the shortest lifespan and balanced HSC are intermediate between My-bi and Ly-bi HSC.

We hypothesized that My-bi HSC are transformed to generate CML. This is based on the observations that 1) CML is a HSC disease and 2) a hallmark of CML is the overproduction of myeloid and the underproduction of lymphoid cells by the transformed HSC. We know that epigenetic regulation causes My-bi HSC to be long-lived, slow growing HSC with a blunted ability for lymphopoiesis. We reasoned that the same epigenetic regulation would predispose these HSC to have a higher incidence of the bcr-abl chromosomal translocation than other types of HSC.

Body

Our hypothesis is strengthened by recent data from our lab showing that the My-bi HSC accumulate when the animals age (Fig.1). Human hematopoiesis becomes increasingly myeloid-biased in adults and CML is a disease of late middle age. Thus, it is tempting to speculate that the increase in My-bi HSC in the aging HSC compartment is responsible for the age-related increase in CML.

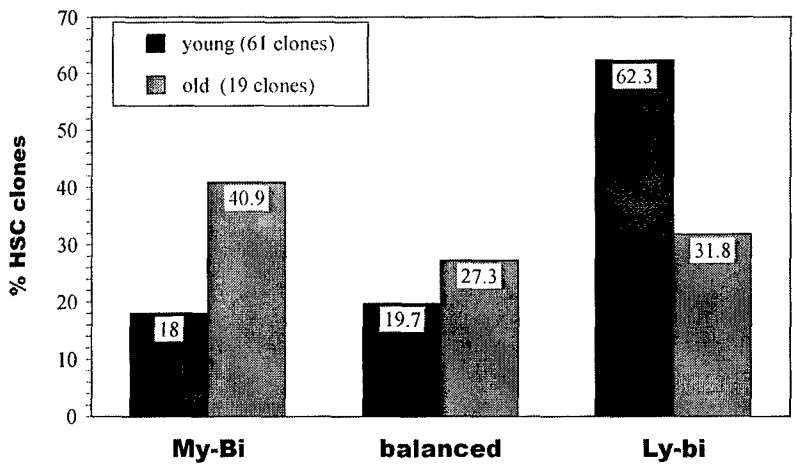

Figure 1. Myeloid-biased HSC accumulate and Lymphoid-biased HSC are depleted in the bone marrow of aged animals. Clonal analysis of repopulating HSC was done as described previously (also see Fig.2). The number of clonally derived HSC from young and old animals is indicated in the Figure legend. Young mice were 1 to 6 months post birth and old mice were at least 18 months of age. All mice were on the B6 background. The distribution of HSC from old mice is significantly different from that of young mice ($p=0.03$)

To test the hypothesis, we proposed to identify My-bi, Ly-bi and balanced HSC and infect these different classes of HSC with a retroviral construct encoding the translocated bcr/abl gene product. As detailed in the original application, we expect that My-bi HSC transformed with bcr/abl but not the other types of HSC will give rise to myeloid-biased CML disease in vivo. The first step was to:

A: Establish hosts repopulated with lineage-biased stem cells (HSC) using in vivo limiting dilution repopulating experiments with Sca-1+Lin- HSC.

Unfortunately, these experiments were significantly delayed since DOD approval for animal use was not obtained until October 18, 2004. Since then, we have sorted Sca-1+Lin- cells to enrich for HSC and injected limiting numbers of these cells. The limiting dilution approach is necessary because lineage-biased HSC can only be detected on the clonal level. When several HSC are injected at the same time, the combined output of mature cells will always be balanced, masking the presence of lineage biased HSC. Because of the dynamical nature of the HSC differentiation program, the earliest that the animals can be evaluated is 5 months after transplantation. Currently, there are 2 experiments

that can be evaluated and representative data are shown in Fig. 2. As expected, My-bi HSC represent a minority of all HSC analyzed. Accordingly, and as proposed we will continue these experiments until we have sufficient numbers of My-bi and Ly-bi HSC clones for the proposed experiments. Several additional sets of repopulated animals have been generated and we expect to have sufficient numbers of My-bi and Ly-bi HSC shortly.

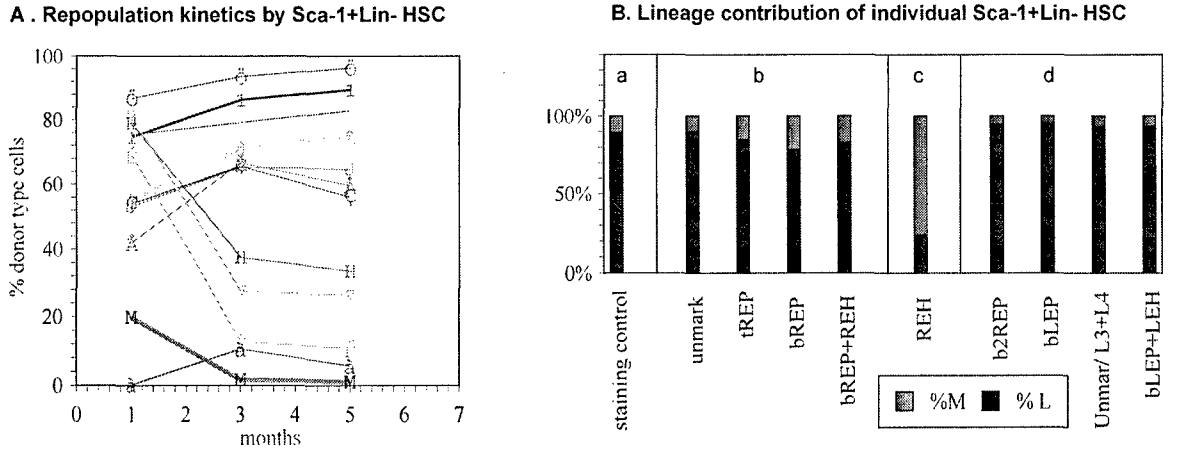

Figure 2: Clonal repopulation patterns of Sca-1+Lin- stem cells. Flow cytometry was used to enrich HSC as Sca-1+Lin- cells about 100-fold. 800 of these Sca-1+ Lin- cells were injected into individual lethally irradiated (2 doses of 550 rds, 2 hrs apart) B6 hosts. Animals were bled bi-monthly at the time points indicated and the number of donor type cells was measured. The kinetics of repopulation by individual HSC clones is shown in panel (A). Each line represents an individual HSC clone. Panel B shows the fraction of lymphocytes and myeloid cells of donor type at 5 months after transplantation. In this set of animals, four balanced (b), one My-bi (c) and four Ly-bi HSC (d) clones were identified.

The next step in the plan as funded was to:

B: Amplify plasmids containing the BCR/ABL p210 and p185 forms and produce high titer retrovirus

We now have sufficient quantities of high quality plasmid DNA for the proposed studies for both the bcr/abl p210-form and control plasmid containing GFP only. We have initiated a careful series of experiments designed to define the conditions that lead to high titer retrovirus production. Because of the nature of the vector [3]transfection efficiency was monitored by quantifying cells that expressed GFP. First, we tried different products to transfect the producer cell line (Clonfectin, Lipofectamine, Superfectin, and Calphos). Of these, the calcium phosphate based method worked best. Next, optimal parameters for (producer) cell density, DNA concentration, length of transfection time, and length of time after transfection for maximum expression were established. An example is shown in Table 1.

Table 1: Defining optimal conditions for retrovirus production.

DNA concentration	% GFP+ cells
2 µg	12.3
4 µg	44.5
6µg	60.7

The indicated amount of highly purified vector DNA was added to 35 mm dish of subconfluent ($8x10^5$) producer cells. Cells were incubated with DNA and Calphos for 10hrs. Thereafter the medium was changed to normal growth conditions. The percent of GFP+ cells was measured 44 hrs later by Flow cytometry. The data indicate that higher concentrations of DNA lead to better transfection efficiency.

Key Research Accomplishments:

1. Established optimal conditions for producing retrovirus
2. Generated lineage-biased stem cells

Reportable Outcomes: NONE

Conclusions: As originally proposed we have begun to generate linage biased HSC and have worked out the conditions to make retrovirus. As proposed, the next step is to infect different types of HSC with bcr/abl and monitor disease outcome.

Personnel Supported by the grant

Libby Virtz, PhD 50%
We were lucky to attract Dr. Virtz to this project in February of 2005. She is a senior postdoctoral fellow with a strong background in molecular biology. Dr. Virtz has produced high quality plasmids and is currently working out the conditions for production of high titer retrovirus production.

REQUEST FOR NO COST EXTENSION

We have requested a 12 month no cost extension to complete the studies.
The project was submitted (and evaluated by the study section) for an 18 months period. However, administrative oversight led to a 12 months grant period. In addition, several unanticipated factors delayed the execution of the project. These included a delay in receiving permission for animal studies from DOD. An unprecedented bacterial infection of the fetal calf serum (FCS) further delayed the execution of the studies. The proposed studies depend on carefully screened FCS and we find that about 1 in 5 lots will work in our assays. The lot of FCS that we purchased was contaminated by low levels of bacteria. The low level of bacterial load together with the unexpected source of contamination caused a lengthy search for the problem and the loss of many experiments. Nevertheless, we are back on track and we anticipate finishing the proposed studies within the next 12 months.

References

1. Muller-Sieburg CE, Cho RH, Thoman M, Adkins B, Sieburg HB (2002) Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. Blood 100:1302
2. Muller-Sieburg CE, Cho RH, Karlsson L, Huang J-F, Sieburg HB (2004) Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. Blood 103:4111

3. Pear WS, Miller JP, Xu L, Pui JC, Soffer B, Quackenbush RC, Pendergast AM, Bronson R, Aster JC, Scott ML, Baltimore D (1998) Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood 92:3780